Uses of Miscanthus Press Juice within a Green Biorefinery Platform

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ABSTRACT

This study assesses **some** uses of nutrient-rich juice mechanically extracted from freshly harvested *Miscanthus x giganteus* (MxG) as part of a green biorefinery system. The juice was used for culturing *Saccharomyces cerevisiae* and lactic acid bacteria. MxG juice was further used as substrate for fermentation to produce lactic acid using *Lactobacillus brevis* and *Lactobacillus plantarum*. The results show that MxG juice was a highly nutritious source for the cultivation of bacteria. Higher concentrations of MxG juice used as culture media, resulted in higher cell growth both aerobically and anaerobically. The highest ethanol yield of 70% theoretical and concentration of 0.75 g/100 ml were obtained from *S. cerevisiae* cultivated with 90% (v/v) MxG juice media and used for miscanthus solid

fraction fermentation. 11.91 g/L of lactic acid was also successfully produced from MxG juice through SSF.

Keywords:

Green biorefinery

Miscanthus x giganteus (MxG)

Saccharomyces cerevisiae

Bioethanol

Lactic acid

1. Introduction

The rapid growth of the world's population as well as the developmental progress being made by a number of emerging economies have both directed to a sharp increase in global energy consumption (Rahman et al., 2015). Volatility in oil prices which affects prudent economic planning, coupled with an escalation in social and industrial awareness

of the environmental impacts associated with the use of fossil fuels, **have necessitated** the need for more sustainable renewable energy options (Rahman et al., 2015).

Resources like wind, solar (photovoltaics), geothermal, ocean (tidal), hydropower and biomass can all contribute to our renewable energy portfolio (Takara and Khanal, 2011). However, biomass represents a more attractive source for the production of both fuels and chemical products, due to its versatility and low environmental impacts (Xiu and Shahbazi, 2015). Biomass is also the only C-rich material source available on earth besides fossil, making it a more viable alternative to fossil resources for the production of transportation fuels and chemicals (Cherubini, 2010).

Analogous to the petroleum refinery, green biorefineries are multi-product systems, which utilize green biomass as an abundant and versatile raw material for the manufacturing of industrial products (Xiu and Shahbazi, 2015). Green biorefineries handle their refinery cuts, fractions and products in accordance with the physiology of the corresponding plant material (Kamm and Kamm, 2004). In green biorefineries, wet-fractionation technology is used as the first step to isolate the content-substances in their natural form, separating the green biomass into fiber-rich press cake and nutrient-rich green juice (Kamm and Kamm, 2007). The green juice contains proteins, free amino acids, organic dyes, enzymes, hormones, other organic substances, and minerals (Kamm and Kamm, 2007). Starting from the green juice, the main focus is directed to products such as lactic acid and its corresponding derivatives, amino acids, ethanol and proteins (Kamm and Kamm, 2007).

In developing a green biorefinery platform, the choice of biomass is important. In this regard, there is a growing interest in using perennial energy grasses as feedstock for biorefinery processes, with MxG, identified as among the best choices for low input bioenergy production in the US (Khanna et al., 2008). MxG is a sterile triploid hybrid of *M. sinensis* and *M. sacchariflorus*, and possesses a C4 photosynthetic configuration, which is an important factor in maximizing the efficiency of conversion of intercepted solar radiation into stored biomass energy, whereby C4 plants are able to use more of the available light energy as well as convert same to stored energy more efficiently than plants with C3 pathways (Heaton et al., 2004).

As a perennial grass, MxG offers several significant advantages such as rapid growth (over 3m tall) in poor soils with minimal water and fertilizer needs, and high lignocellulosic-based carbohydrates (Liu et al., 2013). Due to its high yield and low input demand, many analyses conclude that MxG in the temperate zone is the bioenergy crop that seems able to deliver the highest net GHG mitigation, important for bioenergy sustainability goals (Jørgensen, 2011). Depending on the time of harvest, the moisture content of MxG can range from 70% (fresh weight basis) to 20%, with most of the drying, normally taking place during spring (Lewandowski et al., 2000). To extract protein juice from MxG, a screw press may be used easily (Lewandowski et al., 2000).

In order to make a green biorefinery platform cost effective, it is important to generate valuable final products or other intermediary products that could be used as feedstocks for further downstream processes from both the press cake and the press juice. Particularly, research focus into the use of the press juice is on the increase now, as

processes have been developed for using the press cake for making fuels such as bioethanol (Rahman et al., 2015). Various uses of juice extracted from some lignocellulosic biomass and other types of biomasses have been investigated.

As part of a biorefinery approach, Takara and Khanal (2010), dewatered banagrass using a screw-press, and used the extracted juice for the cultivation of an edible fungus. Rahman et al., (2015) also used cattail juice as a nutritional supplement for microalgal growth and observed that the juice media held great potential as alternative growth media for microalgae and bacteria. A research group also extracted juice from chopped orchard grass and switchgrass biomasses using a screw-press and produced leaf protein from the juice as a co-product of cellulosic ethanol production (Kammes et al., 2011). Grass silage juice was also treated with membrane based and displacement chromatography to manufacture a lactic acid and an amino acid enriched-solutions (Ecker et al., 2012).

Furthermore, juice has been pressed from Italian rye grass, clover grass and alfalfa for the production of lactic acids and other value added products using a green biorefinery platform concept (Andersen and Kiel, 2000). Mannitol-rich ensiled grass press juice has also been applied as a sole carbon source for polyhydroxyalkanoates (PHAs) production, through a high cell density cultivation process (Cerrone et al., 2015). Crude plants juices of desert plants were used exclusively and successfully for culturing rhizospheric microorganism and not as a mere supplement to synthetic culture media (Nour et al., 2012).

The overall goal of this research was to assess various uses for juice extracted from MxG. Particularly, we assessed the viability of MxG press juice for culturing

Saccharomyces cerevisiae and lactic acid bacteria (LAB) as a substitute for expensive commercial media and for producing lactic acid, as part of a green biorefinery platform, using the cultured microorganisms for ethanol and lactic acid fermentation processes respectively. *Lactobacillus brevis* was chosen because it is a well-known heterofermentative strain that has the ability to metabolize monomeric sugars such as glucose and xylose (Cui et al., 2011). Furthermore, *Lactobacillus plantarum* was selected due to its versatility as a result of its facultative heterofermentative nature, allowing it to produce lactic acid through two different metabolic pathways (Zhang and Vadlani, 2015). A combination of the two lactic acid bacteria chosen have been used to effectively produce lactic acid (Zhang and Vadlani, 2015). To the best of our knowledge, these uses of juice from MxG have not been investigated yet. Achieving this will enhance the economic viability and the environmental sustainability of the green biorefinery platform by eliminating a possible waste product, by using it to generate other useful products.

2. Materials and Methods

2.1 Miscanthus harvest and processing

MxG was harvested from NCA&TSU farm in Greensboro, North Carolina in 2015, during early summer. A Tanaka TPH 270s-pole hedge trimmer was used for the harvest, to enable consistent cuts. A DR Wood chipper/shredder (14.50 Pro Manual Start, DR Power Equipment, Vergennes, Vermont) was then used to shred the MxG into smaller sizes. The shredded biomass was pressed and separated into a green juice liquid fraction and a solid cake fraction, using a bench scale Carver press (#2094 Cage Equipment, Carver Inc.,

Wabash, IN) at an optimized force of 30,000 lbs for a residence time of 15 min. The green juice was stored in a freezer at -20 °C until use. The solid fraction was dried in an isotherm oven (Fisher Scientific, USA) at a temperature of 105 °C for 24 h, after which a rotary knife mill (Thomas Model 4 Wiley mill, Thomas Scientific, Swedesboro, NJ) was used to ground the MxG into particle sizes between 0.3 and 0.6 mm for further analyses and downstream processing. Figure 1 illustrates the experimental route taken in this work, including mechanical separation, microorganism cultivation using MxG press juice, and lactic acid and bioethanol fermentations.

2.2 Biomass analytical procedures

The solid and liquid fractions of the *Miscanthus* biomass were analyzed for the ash content, the solid content and their carbohydrate structural constituents (cellulose, hemicelluloses and lignin) using laboratory protocols, LAP #'s 001, 003 and 005, developed by the National Renewable Energy Laboratory (NREL).

The elemental analyses for carbon (C), hydrogen (H), and nitrogen (N) contents were determined by a Perkin-Elmer 2400 II CHNS/O analyzer (Waltham, MA). The oxygen content was calculated by difference: $O\% = 100 - (C + H + N + S)\%$. The *miscanthus* samples were digested with HNO_3/HCl in a microwave oven (200°C, 2MPa) and analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (ARC 3560, Waltham, MA, US) for various minerals.

Compounds in MxG juice was determined using LC/MS. The samples were analyzed using a UPLC-QTOF-MS system (ACQUITY UPLC-SYNAPT MS, Waters

Corp., Milford, MA). An ACQUITY UPLC BEH C18 1.7 μ M VanGuard pre-column and an ACQUITY UPLC BEH C18 1.7 μ M analytical column were used for the analyses at column temperature of 40°C and a flowrate of 0.4 (mL/min). The system was operated in both electrospray ionization (ESI) positive (mobile phases: 0.1% formic acid in water and 0.1% formic acid in acetonitrile) and negative modes (mobile phases 1mM ammonium fluoride in water and acetonitrile) to provide comprehensive coverage. Metabolite annotation for UPLC-QTOF-MS analysis was performed by comparing mass spectra and retention time of each detected signal from the MxG samples to those of reference standards in an in-house library, which contained 500+ endogenous metabolites and plant metabolites.

The concentration of monomeric sugars, including cellobiose, glucose, arabinose and xylose in all liquid fractions as well as the concentration of ethanol were all determined using a Dionex Ultimate 3000 (UHPLC) (Thermo Fisher Scientific, Bannockburn, IL) equipped with a Shodex Sugar SH 1218 ion exclusion column and a Shodex RI-101 refractive index detector. The mobile phase was 0.01N H₂SO₄ at a flowrate of 1 ml/min. The temperature of the detector and column were maintained at 50 °C and 75 °C respectively. All experiments and analyses were performed in duplicate.

2.3 Culture growth and measurements

The growth of *Saccharomyces cerevisiae* (ATCC 24858), *Lactobacillus plantarum* (ATCC 14431) and *Lactobacillus brevis* (ATCC 367) in MxG juice was compared to their growth in commercial growth media (YM broth for *S. cerevisiae* and MRS broth for the

lactic acid bacteria (LAB)). MxG juice concentrations of 90% (v/v), 50% (v/v), 20% (v/v) and 10% (v/v) were tested as culture media for the growth of the selected microorganisms. MxG juice used for the growth of the microorganism was sterilized at 121 °C for 15 mins using Auto clave SQ 500 °C (Yamato Scientific America, Inc., Santa Clara, CA, USA).

The *Saccharomyces cerevisiae* cells for inoculation were cultivated aerobically in Difco YM broth (Becton, Dickinson and Company, Sparks, MD, US) for 24 hours in an I26 Incubator shaker series (New Brunswick Scientific Co. Inc., Edison, NJ, US) at 35 °C and 200 rpm. LAB cells were also cultivated aerobically in Difco Lactobacilli MRS broth (Becton, Dickinson and Company, Sparks, MD, US) at 30 °C, 200 rpm for 24 hours in the incubator shaker. It is from these stocks that the seed cultures for culturing in the MxG juice were taken.

In preparing the MxG juice media and culture growth, a total volume of 100 mL was used, made up of MxG juice, deionized water and 10mL of seed culture (e.g. 90% (v/v) = 90 mL of MxG juice + 10 mL of seed culture; 50% (v/v) = 50 mL of MxG juice + 40mL of deionized water + 10mL of seed culture). Commercial media, YM broth and MRS broth were prepared as directed by the manufacturers and also inoculated with 10 mL seed culture, to give a total volume of 100 mL, serving as controls to the MxG juice media.

After inoculation with the MxG juice media and the control media, the cultures were incubated in an I26 Incubator shaker series (New Brunswick Scientific Co. Inc., Edison, NJ, US) at 35 °C for *S. cerevisiae* and 30 °C for the LAB, under shaking at 200 rpm for 24 hours. Batch cultivation was performed under aerobic and anaerobic conditions using 250 mL baffled base TriForest vented DuoCap Polycarbonate Erlenmeyer Shaker

flasks (TriForest Enterprises, Inc., Irvine, CA, USA), ideal for both aerobic and anaerobic cell culture. Aliquots were taken at hours 0, 12 and 24 to measure optical density and to count colonies.

Quantification of viable cells was carried out by means of a plate count technique on Difco YM agar (Becton, Dickinson and Company, Sparks, MD, US) for *S. cerevisiae* and MRS agar for LAB. YM agar was prepared as directed by the manufacturers. In making MRS agar, MRS broth was prepared as directed by the manufacturers and supplemented with agar (15.0 g/L) (Fisher Scientific, Fair Lawn, NJ, US). To determine the appropriate amount of cell sample to plate, serial tenfold dilutions were prepared using the initial stock cultures that were used for the inoculation of the MxG juice media and the commercial control media. 0.1 mL, 300 mL and 1000 mL were plated in duplicates and incubated at 35 °C for 48-72 h in a CO₂ Jacketed Incubator (Revco Technologies, Asheville, NC, US) and manually counted. At the end of this preliminary run, based on the results, the MxG juice media samples and the controls were plated at the 5th serial dilution, with sample volume 300 mL. These were plated in duplicates and incubated with the same conditions described above, and manually counted. The results were expressed as colony-forming units per mL (cfu/mL).

Optical densities at 600 nm for the samples were also measured every sampling period, using a Thermo Scientific GENESYS 20 Spectrophotometer (Waltham, MA). This spectrophotometric method was carried out to corroborate the yeast colonies counting method. Optical density is the most commonly used technique for estimating chromophore formation and cell concentration in liquid culture (Myers et al., 2013). It is used to

routinely identify the stage of growth for culture induction, inoculation and harvest. This visually-assessed turbidity of a culture is only a rough estimate of the cell concentration (Myers et al., 2013). The applicability of OD-based correlations is however, highly dependent on the measurement specifications and culture conditions (Myers et al., 2013).

All the experiments were carried out inside a Cleanprep PCR Workstation (Dead Air Box) (Mystaire Inc., Creedmoor, NC, US), equipped with UV light and designed to provide sterilization of instrumentation before beginning a process and providing an enclosed working environment during the process. Experiments were carried out in quadruplicates to offer greater accuracy of results.

2.4 Fermentation of MxG solid fraction to produce ethanol

MxG solid cake was taken through simultaneous saccharification and fermentation (SSF) to produce ethanol, using *S. cerevisiae* microorganisms grown aerobically with MxG juice media. MxG solid fraction processed as described in 2.1 above was first taken through dilute sulfuric acid pretreatment using a Dionex ASE 350 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, C.A), with pretreatment conditions of 1% (v/v) sulfuric acid solution, 160 °C pretreatment temperature and 10 mins pretreatment residence time.

For SSF, a pretreated biomass loading of 5 g (wet basis) with a total working volume of 50 mL and a pH adjusted to 4.5 by the addition of 0.05 M citric buffer was used. Wheaton septum glass bottles (125 mL) were used as fermentation reaction vessels. A cocktail of enzymes including cellulose (Novozyme NS 50013) at a loading of 60 FPU/g

glucan, hemicellulase (Novozyme NS 22002) at a loading of 2.5 FBG/g glucan and β -glucosidase (Novozyme NS 50010) at 4.5 CBU/g glucan was used for enzymatic hydrolysis. *Saccharomyces cerevisiae* (ATCC 24858) was then added to the reaction vessel to begin the SSF process.

Saccharomyces cerevisiae (ATCC 24858) was cultivated using MxG juice, with 10 mL of the seed culture used to inoculate 90 mL of the MxG Juice media in a 250 mL Erlenmeyer flask. The cultures were incubated in a shaker at 35 °C and 200 rpm and grown aerobically for 24 hours. The suspended yeast cultures were then transferred into 50 mL capped centrifugation tubes, and harvested using a Centra-GP8R centrifuge (ThermoIEC, Needham Heights, MA, US) at 2600 RCF for 15 min at room temperature. Afterwards, the supernatant was discarded and the cells were washed with peptone water after which they were transferred into the 125 mL Wheaton septum glass bottles containing the pretreated hydrolyzate already inoculated with the enzymes. Fermentation occurred under aerobic conditions, with the fermentation cultures placed in a rotary shaker and incubated at 35 °C and 200 rpm. Aliquots were collected at hours 0, 3, 6, 9, 12, 24, 48, 72 and 96, and analyzed for sugars and ethanol concentration by way of an HPLC. *Saccharomyces cerevisiae* cultured with YM broth was also used for SSF to serve as control and allow for comparison. The ethanol yield was expressed as the percentage of the theoretical yield using the following formula;

$$\% \text{ Yield}_{\text{ethanol}} = \left[\frac{C_{\text{ethanol},f} - C_{\text{ethanol},i}}{0.568f \cdot C_{\text{biomass}}} \right] \times 100\% \quad (1)$$

Where $C_{\text{ethanol},f}$ is the ethanol concentration at the end of the fermentation (g/L), $C_{\text{ethanol},i}$ is the ethanol concentration at the beginning of the fermentation (g/L), C_{biomass} is the dry

biomass concentration at the beginning of the fermentation (g/L), f is the cellulose fraction of the dry biomass (g/g), and 0.568 is the conversion factor from cellulose to ethanol. All experiments were carried out in duplicates.

2.5 Lactic acid production from MxG Juice

Lactic acid fermentations were carried out with fresh, unsterile miscanthus juice. Lactic acid fermentation with unsterilized juice where the content of naturally occurring plant enzymes is intact is preferred (Andersen and Kiel, 2000). The juice was inoculated with freshly grown *L. plantarum* and *L. brevis*. Three different experimental combinations were tried, 1) *L. plantarum* only fermentation, 2) *L. brevis* only fermentation and, 3) *L. plantarum* + *L. brevis* fermentation. Fermentations were carried out using 250 mL Wheaton septum bottles as reaction vessels. A ratio of 1:2 (v/v) was used for the experiments with 50 mL of MxG juice to 100 mL of fermentation broth. 0.05 M citrate buffer was used to maintain a pH of 4.5. A combination of Cellic Ctec (Novozymes CZP0005) with a dosage of 60 FPU/g glucan and xylanase (Novozymes NS-50030) with a dosage of 500 FXU/g glucan was used for enzymatic hydrolysis as part of a simultaneous saccharification and fermentation process. The fermentations were carried out aerobically in an incubator at 35 °C and shaking at 160 rpm. Samples were taken at hours 0, 6, 9, 12, 24, 48, 72, 96, 120, and 144.

3. Results and Discussion

3.1 Compositions of miscanthus cake and juice

After mechanical pressing, the mass distribution of MxG was about 0.4 g/g of solid and 0.5 g/g of liquid (Boakye-Boaten et al., 2016). The total solid content of MxG press cake was 17.4 % of the total biomass, and the solid content of the juice was 0.1 % (Table 1). The various compositions of the MxG cake and juice are presented in Table 1. As expected, the carbohydrate compositions of the cake are higher than those of the juice, with the cellulose content of the juice for instance, being lower than that of the cake because, cellulosic fibers are less extractable as a result of the polysaccharide matrix structure in biomass (Rahman et al., 2015). The elemental compositions of the two fractions are slightly comparable, with the juice fraction showing a higher amount of crude protein content than the solid cake as some partial proteins found in the raw miscanthus were likely extracted into the juice.

The higher content of ash in the juice is due in part to the greater content of some inorganic chemicals in the juice compared to the cake. ICP analysis showed that the most dominant elements in the MxG samples were Calcium (Ca), Potassium (K), Magnesium (Mg) and Sodium (Na). With the exception of Na, the juice fraction contained higher amounts of these dominant elements than the solid fraction. In most cases, the juice fraction further contained higher amounts of the least dominant elements than the solid cake fraction (Table 1). LC/MS analysis conducted on the MxG juice (Table 2), also revealed the presence of various amounts of organic acids, amino acids and metabolites, which are useful as supplementary feed for the growth of microorganisms (Rahman et al., 2015).

3.2 Aerobic and anaerobic growth of *S. cerevisiae*

After conducting aerobic and anaerobic growth experiments using the MxG juice as the growing media, it was observed that generally, the higher the concentration of the juice used for the growth, the higher the amount of yeast colonies counted (Figure 2a). For aerobic growth of *S. cerevisiae*, 90% (v/v) MxG media exhibited the highest amount of yeast colonies formed of about 1.2×10^8 cfu/ml after 12 hours and 1.6×10^8 cfu/ml after 24 hours, performing better than the commercial YM media. Growth with the 50 % (v/v) MxG media was comparable to growth with the commercial media after 24 hours (Figure 2a). The higher growth rate at higher concentrations can be attributed to the availability of more minerals and other components in the juices at those concentrations, which stimulated the growth (Survase et al., 2013).

Improvements in the cultivation and growth of microorganisms have been substantially achieved by the inclusion of plant infusions and extracts (Nour et al., 2012). The presence of amino acids and vitamins, as growth factors in the juice extract, aided the growth of the microorganisms under both aerobic and anaerobic conditions (Nour et al., 2012). The results show that the crude MxG juice extracted, contained sufficiently rich nutrients that successfully and solely supported the growth of *S. cerevisiae*. Work done by Corona et al., (2016) indicates that both yeasts and lactic acid bacteria were capable of growing in juice extracted from vegetables. Various volatile organic chemical compounds including acids, alcohols, aldehydes, esters among others, present in the juice extracts assisted in the growth of the microorganisms (Corona et al., 2016). Alfalfa juice has also been tested as a nitrogen source for the growth of *C. Acteobutylicum*. The results showed

an absence of the lag phase during the growth of the microorganism, which suggested that the microorganism was able to easily adapt to the medium supplemented with alfalfa juice (Mechmech et al., 2015).

Results obtained for the anaerobic growth experimentation was similar to the results above in terms of the concentrations of the MxG juice used as growth media for *S. cerevisiae*, with 90% (v/v) showing a higher number of cell number after 12 and 24 hours, than the commercial media (Figure 2b).

A comparison of aerobic and anaerobic growths showed that in all cases, aerobic growth performed better in cell numbers formed after 12 and 24 hours than the anaerobic growth (Figure 3). At an MxG juice concentration of 50% (v/v) for instance, the number of cells formed after 24 hours during aerobic growth was about 1.0×10^8 cfu/ml, while anaerobic growth at the same concentration was about 8.0×10^7 cfu/ml (Figure 3). A majority of ascomycetous yeasts are strictly aerobic and cannot be propagated at low oxygen levels (Moller et al., 2001). *Saccharomyces cerevisiae* however, is a petite-phenotype-positive yeast, which is known to successfully grow in the absence of oxygen, unlike other yeasts such as *Kluyveromyces lactis* and many other yeasts which are petite-negative and cannot grow anaerobically (Moller et al., 2001). Several observations show that the most probable progenitor of the modern *Saccharomyces* yeasts, as well as other related genera, is likely a petite-negative and aerobic yeast, making aerobic growth of these microorganisms much more successful (Moller et al., 2001).

In the presence of oxygen, *S. cerevisiae* can undergo aerobic respiration, where glucose is broken into CO_2 and ATP is produced by protons falling down their gradient to

an ATPase (Bekatorou et al., 2006). When oxygen is lacking, *S. cerevisiae* can only get their energy from glycolysis and the sugar is instead converted into ethanol, making anaerobic respiration a less sufficient process than aerobic respiration (Bekatorou et al., 2006), and as such a higher number of cell formation under aerobic growth conditions.

Previously, species of 75 yeast genera were examined for their ability to grow anaerobically in complex and mineral media. It was observed in this study that *S. cerevisiae* was about the only yeast species for which good anaerobic growth was realized, a property the authors described as very rare among yeast (Visser et al., 1990). A general limitation for the anaerobic growth of yeasts other than *S. cerevisiae* even on the most common of substrates, is a sufficiently high rate of ATP formation (Sonderegger et al., 2004).

In figure 4a, the growth curve shows that aerobic yeast growth in 90% (v/v) concentration of juice was about two times higher than the controls (YM medium). The same was observed for anaerobic growth (Figure 4b). The observed growth curves are consistent with literature, given the period of time under consideration. In the early part of the time-course, for both aerobic and anaerobic growth, there is a slight lag as the microorganisms become aware of their environment and the availability of nutrients for their growth. Thereafter, there is an exponential increase in cell density as a result of the rapid division of the cells, followed by a scarcity of nutrients which results in the growth rate of the microorganisms, slowing down as the yeast adjust to their metabolic machinery.

Furthermore, after both aerobic and anaerobic growth, the cells were harvested, dried and weighed, with the results showing a greater harvest of cells for 90 % (v/v) MxG media compared to 50% (v/v) MxG media and the commercial YM media (Table 3).

3.3 Aerobic and anaerobic growth of lactic acid bacteria (LAB)

Miscanthus juice was also used to successfully cultivate *Lactobacillus brevis* and *Lactobacillus plantarum*. For lactic acid bacteria growth, MRS media, the commercial media used as a control, was comparable to the highest concentration of juice (90 % v/v) in terms of the amount of microbial cells cultivated over the period. It can be seen from figure S1a that after 24 hours, 90 % (v/v) juice concentration media for the aerobic growth of *Lactobacillus brevis* performs only slightly better than the growth with the commercial media (MRS media). A similar trend was observed for the anaerobic growth of *Lactobacillus brevis*. Growths of *Lactobacillus plantarum* (Figure S1b) were comparable in trend to what was observed for *Lactobacillus brevis*. Generally, *S. cerevisiae* grew better in the MxG juice than the lactic acid bacteria strains tested.

Lactic acid bacteria (LAB) are considered oxygen-tolerant anaerobes, with growth conditions and type of metabolism, significantly affecting the stress responses in LAB (Zotta et al., 2014). Conditions which promote aerobic and respiratory growth in LAB, such as the presence of oxygen and vitamins in the media is reported to induce useful traits in LAB, such as stress tolerance, making these bacteria useful for industrial and biotechnological applications (Zotta et al., 2014). Zotta et al., (2014) showed that for most LAB strains able to cope with aerobic conditions, there was an increase in cell production

compared to anaerobic cultivation. This is consistent with what was observed in our current study.

It is also agreed that *Lactobacillus plantarum* is capable of an O₂ dependent metabolism (Murphy and Condon, 1984), consistent with the results of the aerobic growth of *L. plantarum* observed in this work. This bacteria is a heme-protein oxidase, which reduces O₂ during metabolism, however it is not clear if this is ultimately beneficial or detrimental to *L. plantarum* cells cultivated aerobically (Murphy and Condon, 1984). Further studies have shown that the cell yield of *Lactobacillus plantarum* was higher under aerobic conditions than under anaerobic conditions, during the production of lactic acid from lactose (Gupta et al., 2011). The differences in growth can be attributed to differences in metabolic pathways under aerobic and anaerobic conditions (Murphy and Condon, 1984). Generally, the increase in cell numbers in the presence of oxygen is due to the generation of additional ATP (Gupta et al., 2011).

Furthermore, studies have shown that aerobic cultivation of *Lactobacillus brevis* increases their oxidative stress (Zhao et al., 2014). In a study, two strains of *Lactobacillus brevis* used for experimentation, both grew better under aerobic conditions (Zhao et al., 2014). This is consistent with our results. In some cases however, some strains of *Lactobacillus brevis* have been found to grow poorly under stationary and anaerobic conditions (Stamer and Stoyla, 1967).

3.4 Fermentation of MxG solid fraction to produce ethanol

Dilute sulfuric acid pretreatment of the miscanthus was effective in changing the composition of the biomass, resulting in an increase in the cellulose and lignin contents, and a decrease in the hemicellulose content of the biomass samples (Table S1). Due to the complete removal of hemicellulose during the pretreatment process, high enzymatic digestibilities were observed. High enzymatic digestibility and the use of a cocktail of enzymes for the SSF process resulted in high theoretical ethanol yields (Boakye-Boaten et al., 2016). As indicated earlier (Table 3), the mass of microorganisms harvested after aerobic growth in 90 % (v/v) MxG juice media was higher than that harvested after aerobic growth in the YM commercial media. This implies that more microorganisms were available for fermentation after growth in 90% (v/v) MxG juice than available from YM growth. The availability of more microorganisms for fermentation likely resulted in higher ethanol concentrations (Figure 5) and theoretical ethanol yields (Figure 6) for SSF fermentation using organisms cultivated with 90% MxG juice.

3.5 Lactic acid production from MxG juice

The extracted juice from MxG was used successfully for lactic acid fermentations with *Lactobacillus plantarum*, *Lactobacillus brevis* and a combination of the two strains (Table 5). Fermentation with a combination of *Lactobacillus plantarum* and *Lactobacillus brevis* gave the highest lactic acid concentration of 11.91 g/L after 24 hours. This could be attributed to the efficient utilization of both the cellulose and hemicellulose fractions present in the extracted juice (Table 1). Andersen and Kiel (2000) produced comparable and slightly higher concentrations of lactic acid of 11.0 to 15.0 g/L using brown juice

extracted from grass, with *L. salivarius* and *L. paracasei* as fermenting organisms. On a larger scale, about 22.0 g/L lactic acid has been produced from silage juice in a pilot plant (Ecker et al., 2012). Cui et al., (2010) observed that the used of mixed cultures or co-cultures of lactic-acid producing microorganisms may increase the conversion efficiency of substrates. A combination of homo and hetero fermentative strains of lactic acid bacteria makes the fermentation of both hexoses and pentoses possible (Cui et al., 2011). Ethanol and acetic acid were observed as byproducts in smaller quantities during the fermentation process. Fermentation with *L. plantarum* alone resulted in the highest ethanol concentration of 3.61 g/L, possibly due to heterolactic fermentation. Co-fermentation with *L. plantarum* and *L. brevis* resulted in 0.29 g/L ethanol concentration. Furthermore, acetic acid was observed in quantities ranging from 0.66 g/L to 0.78 g/L for *L. plantarum* fermentation, 0.63 g/L to 0.81 g/L for *L. brevis* fermentation and 0.63 g/L to 1.38 g/L for co-fermentation using *L. brevis* and *L. plantarum*.

4. Conclusions

MxG juice uses were assessed as part of a green biorefinery platform, producing ethanol and lactic acid using microorganisms cultured within the platform structure.

Results showed the juice was highly nutritious and supported the growth of the microorganisms and bacteria. Aerobic cultivation produced higher cell numbers in most cases than anaerobic cultivation for various reasons. Furthermore, mixed culture fermentation of the juice produced the highest concentration of lactic acid after 24 hours.

These uses of the MxG juice eliminate the extracted juice as a waste stream within a green biorefinery platform, making the concept more economical and environmentally friendly.

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Figure

Figure 1. Processing diagram describing some uses of *Miscanthus x. giganteus* solid and liquid fractions as part of a biorefinery platform

Figure 2a. Aerobic growth of *Saccharomyces cerevisiae* using YM broth and miscanthus juice as media

Figure 2b. Anaerobic growth of *Saccharomyces cerevisiae* using YM broth and miscanthus juice as media

Figure 3. A comparison of aerobic and anaerobic growth of *Saccharomyces cerevisiae* using YM broth and miscanthus juice at various concentrations

Figure 4a. *Saccharomyces cerevisiae* growth under aerobic conditions

Figure 4b *Saccharomyces cerevisiae* growth under anaerobic conditions

Figure 5. Ethanol concentration from SSF fermentation of MxG solid fraction using *S. cerevisiae* grown in different culture media

Figure 6. Highest theoretical ethanol yield from SSF of MxG solid fraction using *S. cerevisiae* grown in different culture media

Table 1

Characteristics of MxG cake and juice

Group/specific	Miscanthus Cake	Miscanthus Juice
Total Solids, wt%	17.4	0.1
Ash, % dry matter	1.8	3.9
H ₂ O, wt%	82.6	99.9
Biomass composition of dry matter, wt %		
Cellulose	63.7	39.2
Hemicellulose	49.1	19.7
Lignin	22.1	12.3
Elemental Composition (%)		
C	48.2	37.8
H	6.6	5.2
N	0.4	0.8
S	0.5	1.0
O	44.3	55.2
Protein content (%)	2.5	5
Mineral Composition (ppm)		
Al	21.3	39.3
B	52.0	95.3
Ca	412.3	1968.2
Cd	2.7	7.4
Cr	38.0	19.6
Cu	25.1	20.1
Fe	97.4	76.2
K	5506.6	93083.3
Mg	1901.3	19658.3
Mn	30.8	251.3
Na	1301.0	487.1
Ni	7.5	5.1
P	84.2	475.7
Pb	7.0	10.3
S	11.0	23.2
Zn	22.9	39.7
Si (g/ml)	2926.4	273.6
Mo (g/ml)	11.5	6.9
Ba	BDL	BDL
Sr	BDL	0.01

Table 2

LC/MS analysis of MxG juice

Compound Name	Ret Time (min)	m/z
3-hydroxypropionic acid	0.6756	89.0
7-methylxanthine/3-methylxanthine	1.4121	167.1
Adenine	0.8119	136.1
aminobutyric acid	0.6186	102.1
apigenin/genistein	3.332	271.0
Arginine	0.7322	175.1
azelaic acid	2.9221	211.1
Biotin	2.2158	227.1
caffeic acid	2.2282	181.1
Citrulline	0.7176	159.1
Cytosine	0.6817	112.1
fructose/mannose	0.6829	179.1
fumaric acid	0.5659	115.0
Genistin	2.392	433.1
gibberellic acid	2.7087	347.2
glucaric acid	0.6975	233.0
gluconic acid	0.6487	195.0
glutamic acid	0.8481	148.1
histidine	0.686	154.1
Hypotaurine	0.5547	110.0
Hypoxanthine	1.0334	137.0
Leucine	1.2646	132.1
l-tyrosine	1.1882	182.1
m-Couraric acid	2.6982	167.1
methionine	0.9698	133.0
nicotinic acid	0.9439	124.0
Pantothenate	1.6058	220.1
Paraxanthine	1.9946	181.1
phenylalanine	1.506	166.1
phosphoenolpyruvic acid	0.6185	167.0
Proline	0.7951	116.1
Riboflavin	2.1214	377.1
succinic acid	1.1895	119.0
Thymine	1.413	127.0
Tryptamine	2.0723	144.1
tryptophan	1.794	188.1
Uracil	0.8922	113.0

Table 3

Weight of harvested *S. cerevisiae* cells

Media	Weight (g)
Aerobic Growth	
90% (v/v)	0.92 ± 0.5
50% (v/v)	0.48 ± 0.1
YM Media	0.67 ± 0.05
Anaerobic Growth	
90% (v/v)	0.77 ± 0.1
50% (v/v)	0.41 ± 0.1
YM Media	0.44 ± 0.1

Table 4

Concentration of lactic acid produced from MxG juice

Time (hrs)	<i>Lactobacillus plantarum</i> Fermentation (g/L)	<i>Lactobacillus brevis</i> Fermentation (g/L)	<i>L plantarum</i> + <i>L brevis</i> fermentation (g/L)
0	0.00	0.00	0.00
12	11.60	5.74	11.59
24	11.62	11.59	11.91
48	11.67	10.26	10.20
72	10.79	9.03	6.62
96	8.27	6.36	4.58
120	7.19	5.92	2.44
144	6.97	4.86	4.70